

Black Truffle Production, Soils and Mediterranean Woods: Spanish Research Contributions (1962-2006)

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Abstract: The production of mycorrhizal fungi provides higher economic returns than any other forest product in many Mediterranean woods. In these areas, truffles (*Tuber* spp.) and mushrooms of *Boletus* and *Lactarius* genus are the fungi that yield the highest economic benefits. However, there are still evident gaps in the understanding of the truffle's biological cycles, ecology and interactions with host plants and other fungi. In the last 12 years, Spanish research has begun to respond to the requirements of truffle research. Most of Spanish *Tuber* references deal with the areas of agronomy, forestry, ecology and soil science. In this respect, Spain is one of the main truffle-producing countries in the world and its varied climate, topography and lithology make it a useful geographic area for increasing knowledge on *Tuber* ecology and truffle production. This work describes the content of Spanish research and its impact on the knowledge of truffles.

INTRODUCTION

Ectomycorrhizae are formed as a result of the association between fungi and roots of many plant species. Lian *et al.* and Poma *et al.* explain that ectomycorrhizae enhance uptake and utilization of nutrients by plant roots, strengthen self-defense ability of plants, facilitate development of plants, and improve soil quality. Ectomycorrhizal fungi are important in the forestation and rehabilitation of vegetative ecosystems, the remediation of soil and water loss, and the diversification of biological species. Moreover, Wang and Hall indicate that the most expensive and sought after of the edible mushrooms belong to the mycorrhizal group. The total market for these is measured in billions of US dollars.

In this regard, the production of mycorrhizal fungi provides higher economic returns than any other forest product in many Mediterranean woods. In these areas, truffles (*Tuber* Micheli ex Wiggers) and mushrooms of *Boletus pinophilus* Pilat and Dermek, *B. edulis* Bull.: Fr. and *Lactarius deliciosus* L.: Fr. are the fungi that yield the highest economic benefits. Among the *Tuber* species, the black truffle of Périgord (*T. melanosporum* Vittad.) is especially appreciated for its culinary value due to its intense aroma, its high yield and the prices it commands (more than 1000 €/kg'). *T. melanosporum* is of great ecological and forest value because it inhabits carbonated soils in impoverished mountain ecosystems within the Mediterranean continental climate

Over the past 100 years, harvests of many mycorrhizal mushrooms have declined dramatically, which has prompted interest in the development of methods for their cultivation. Hall and Wang indicate that a few of the ectomycorrhizal fungi can now be cultivated using trees specially infected with the appropriate fungus. Those which command very high prices, like truffles, *Tuber melanosporum*, and matsutake, *Tricholoma matsutake* (S. Ito et Imai) Sing., warrant the expense of establishing plantations dedicated to their production. In fact, successful production of *Tuber melanosporum* is observed in different crops of Europe and other regions, but non controlled factors are still inducing strong heterogeneity in the production from year to year

The biology of *Tuber melanosporum* has been fully studied and described in extensive monographs. However, there are gaps in our knowledge of *T. melanosporum* biological cycle, such as its adaptive responses to the physical environment, factors inducing fruiting, soil influence in truffle development and interactions with host plants and other fungi, etc.

Bencivenga and Chevalier have confirmed that the ecology of *Tuber melanosporum* has not been thoroughly explained and that its study is of great importance to improve harvesting, cultivation and natural protection. Garcia-Montero indicate that the international research over the last 10 years has been very limited in regard to studies carried out in natural environments and on plantations; only 16% of the references deal with the areas of agronomy, forestry, ecology and soil science, while 69% deal with molecular biology and biochemistry. However these authors explain that in the last 12 years, Spanish research has begun

to respond to the requirements of truffle research. Specifically, 69% of the Spanish references deal with the areas of agronomy, forestry, ecology and soil science, and 53% are based on studies done in natural areas and on truffle plantations. In this respect, Spain's varied climate, topography and lithology make it a useful geographic area for increasing knowledge on *Tuber* ecology and truffle production. In this regard, Spain's great variability gives rise to singular habitats with symbiotic plants whose western Mediterranean distribution has made their study more difficult in other countries.

Until 1993, the natural production and cultivation of *Tuber melanosporum* in Spain was relatively unknown, and there was little research being done on the subject; however, this activity has taken on greater importance in recent years. Actually, Spanish production of *T. melanosporum* accounts for between 30% and 50% of production worldwide. Moreover, since 1994 to 2006, Spanish research has produced at least 200 scientific references

In view of the need to expand current knowledge of the ecology of *Tuber melanosporum*, and with the aim of increasing production of this valuable forest resource, we have carried out a synthesis of the last ecological studies on quantitative production of carpophores, soil properties and ecology in different Mediterranean woods situated in Spain. Moreover, we review the Spanish research contributions dealing with the biology, ecology and cultivation of *T. melanosporum* and its impact in the international truffle knowledge. This paper is the second part of a previous paper entitled "Historical summary and reference list of Spanish truffle (*Tuber* spp.) research (1962-2006)", to show the scientific contents of this Spanish reference list.

SOIL FACTORS THAT INFLUENCE *TUBER MELANOSPORUM* PRODUCTION IN CENTRAL SPAIN

Different studies have examined which soil properties have the greatest impact on *Tuber* biology; these include comparisons of the soil requirements of different species of truffles, analyses of soil variability and its relationship with climate, and studies relating soil parameters to production and development of *T. melanosporum*

These authors indicate that *Tuber melanosporum* is strictly calcicolous and grows in calcareous soils with a C/N relationship of close to 10, in which the soil texture is balanced and tends to be simple and well constructed. However, Sourzat [39] indicates that the soil parameters that *T. melanosporum* tolerates are highly variable. Callot Lulli *et al.* [38], Castrignano and Ricard report that the development of *T. melanosporum* increases in deep, well-drained, aerated soils with a reservoir of available water during dry periods. Total organic carbon content must be low in order not to modify their pH, and they require considerable porosity, originated by biological activity (above all, by worms and ants). However, Callot, Ricard and Garcia-Montero highlight the lack of knowledge as to how the physical-chemical properties of fine soil influence truffle development. Moreover, many authors fail also to supply quantitative data on the relationship between soil properties and truffle production.

In this regard, in the last 10 years, we have developed several studies in Central Spain to explain more fully the

impact of surface horizon soil properties on the productivity of *Tuber melanosporum* carpophores, moreover we analyzed the soil interactions with burn development and host truffle plants, particularly rockroses (*Cistus* L.), and we study the impact of soil on the interactions between truffle species inside the *Tuber melanosporum* burns, as *T. aestivum* Vittad., *T. rufum* Pico ex Fries and *T. mesentericum* Vittad. These studies were done in the Alto Tajo Nature Reserve (Peralejos de las Truchas, Guadalajara), a location with several areas of natural populations of *T. melanosporum*

These studies describe that statistical analysis showed that the collective influence of the conventional soil features (granulometric texture, pH, calcareous fractions, organic carbon, total nitrogen, exchangeable cations) on truffle production is low, as PCA explains only 27% of the variance in *Tuber melanosporum* production. However, a high percentage of active limestone (calcium carbonate extractable with ammonium oxalate) accounts for up 43% of *T. melanosporum* carpophore production and 51% of the variance in the size of *T. melanosporum* burns.

Active carbonate is a finely-divided fraction of calcareous rock. It is smaller than 50 μm in size, very chemically active, and constitutes an important reserve of exchangeable Ca^{2+} . Active carbonate maintains high levels of exchangeable Ca^{2+} in the soil because it counterbalances losses from leaching and other processes. Active carbonate and exchangeable Ca^{2+} affects *Tuber melanosporum* in several ways. Ricard indicates that it is difficult to judge the impact of active carbonate on truffle culture because several factors must be considered; he also considers the lack of studies on active carbonate in truffle culture to be an oversight.

In this regard, active carbonate is associated with exchangeable Ca^{2+} content and stoniness abundance, most importantly, active carbonate regulates soil pH and participates in the organisation and maintenance of the soil structure, also, Ca^{2+} is indispensable in the growth of the mycelium of *Tuber melanosporum*, all of which affect the successful productivity of *T. melanosporum* carpophores. Garcia-Montero *et al.* [45] indicate that statistical analysis showed that active carbonate accounted for 42% of the variance in exchangeable Ca^{2+} concentration, and the exchangeable Ca^{2+} abundance explained 20% of the variance in carpophore production. In addition, a significant correlation was found between active carbonate concentration and surface stoniness.

The availability of active carbonate in the soil depends on complex pedologic, climatic and biological factors. The dissolution, transport and accumulation of carbonates in the soil depend on the relationship between water dissolution and gaseous CO_2 , soluble H_2CO_3 and HCO_3^- , and solid CaCO_3 phases. The solubilisation and leaching of carbonates increases with pluviosity, respiration and metabolism of roots and mycelia, and other factors that increase the partial pressure of CO_2 ($p\text{CO}_2$) in the soil. Bicarbonate precipitates in the form of carbonate when it is concentrated in capillary water (evaporated or absorbed by the roots). This also occurs when the $p\text{CO}_2$ diminishes due to aeration caused by macro fauna and the disappearance of the biological respiration of the roots at depth, among other factors

Tuber melanosporum inhabits Mediterranean dry soils, which the fungi dry up by eliminating plants from their burns. The soil water evaporation and the disappearance of roots in the burns therefore favour surface accumulation of secondary carbonate. This also occurs when the CO₂ diminishes due to aeration. In this regard, *T. melanosporum* is closely related to the aeration of the soil

Our results indicate that the development of the *T. melanosporum* burn encourages the formation of a large amount of active carbonate and exchangeable Ca²⁺. *T. melanosporum* fruition and burn size are simultaneously favoured by a high concentration of both factors, which suggests a feedback process. Finally, these authors indicate that this feedback process provides *T. melanosporum* an additional advantage over its competitors, *T. aestivum*, *T. rufum* and *T. mesentericum*, when active carbonate reaches high soil concentration in the *T. melanosporum* burns. Therefore, active carbonate is a major factor in the fruiting, burn development, and aggressiveness of *T. melanosporum* versus other truffle species.

For this reason, we propose the study of calcareous amendments with fine limestone to maintain the concentration of active limestone in practical truffle culture. However, these conclusions should be interpreted with caution. In fact many other biotic and abiotic factors play an important role in truffle productivity in cultures and natural burns. Ricard

also suggests calcareous amendments of fine limestone in practical truffle culture although he recommends that they should be used with care and in moderation. New studies are required into the effects of active limestone on the biology of *Tuber melanosporum*, *T. brumale* and other truffles into the role played by soil and biological processes in maintaining elevated concentrations of active limestone in *T. melanosporum* cultures and natural burns.

FOREST ECOLOGY AND SYMBIOTIC PLANTS THAT INFLUENCE *TUBER MELANOSPORUM* PRODUCTION IN CENTRAL SPAIN

In truffle culture, the terms "burn", "quemado", "brule" or "pianello" are used to describe spots where truffles grow and refers to truffles' phytotoxic capacity and their ability to create clearings in the vegetation where their carpophores bear fruit. *Tuber melanosporum* burns generally present a circular shape around the symbiotic log, and the vegetation-free surface area spreads outwards at a speed of 10 to 15 cm per year, sometimes reaching areas several dozen metres in diameter

. The environmental conditions created by the burns of *T. melanosporum* are very favourable for the development, mycorrhization and fruition of this truffle. However, Callot, Ricard [16] and Granetti

indicated that the biological and physical-chemical conditions related with burn development have yet to be clarified.

Most studies of *Tuber melanosporum* fail to supply statistical data on carpophore production and other quantitative data associated with its ecology. In view of the need to expand current knowledge of the ecology of *T. melanosporum*, and with the aim of increasing production of this valuable forest resource, we have carried out a study on the quantitative production of carpophores, the size of *T. melanosporum* burns and their soil properties in different woods situated

close together in the Alto Tajo Nature Reserve, in the central region of Spain. This region is a suitable area for the study of *T. melanosporum* since it is a continental elevated region with particular substrates and plants whose relationships with *T. melanosporum* are unknown. This study area has a maximum total production of 500 kg of *T. melanosporum* carpophores.

During 7 years, we studied 208 burns associated with *Quercus ilex* subsp. *ballota*, *Q. faginea*, *Corylus avellana* and *Tilia platyphyllos* in the centre of the Iberian Peninsula

. The results showed that burn size is a very significant factor as it accounts for 38% of the variance in carpophore production. However, no significant difference in the size of burns was observed between five types of wood, although significant variability in carpophore production existed between these wood types. Furthermore, statistical analysis shows that a high concentration of active carbonate in the soil accounts for up to 51% of the variance in *T. melanosporum* burn size. The ecological conditions of burns cause elevated concentrations of active carbonate and exchangeable Ca²⁺, and *T. melanosporum* activity and burn size are simultaneously favoured by a high concentration of both factors, which suggests a feedback process. These results may indicate a possible application for the use of calcareous amendments in natural burns and truffle cultivation.

We also determined the considerable truffle-producing ability and mycorrhizing capacity of *Quercus faginea*. Although *Q. faginea* is a very little-known species in truffle culture, the results provide support for its implementation. Moreover, in these studies we conclude that at present *Pinus sylvestris* L., *P. nigra* Arnold subsp. *salzmannii* (Dunal) Franco, *Tilia platyphyllos* Scop. and *T. x vulgaris* Hayne (= *T. x europaea* auct. p.p.) are of little interest to *Tuber melanosporum* culture, as they hinder carpophore production. However, these studies have also confirmed that pines and lime trees mycorrhize easily with *T. melanosporum*, both in the laboratory and in natural environments. On this basis, we propose that pines and lime trees mycorrhize may act as transmitters of *T. melanosporum*, although they do not induce fruiting. As a result, the commercial cultivation of *Pinus nigra salzmannii*, *P. sylvestris*, *Tilia platyphyllos* and *T. x vulgaris* seedlings mycorrhized with *Tuber melanosporum* is not recommended in truffle culture at the present time [47, 58].

At the moment, there are very few studies on *Tuber melanosporum* associated with *Cistus L.* (rockroses) populations. In Central Spain, we confirm that *C. laurifolius* L. shows carpophore production of *Tuber melanosporum*

. We demonstrate a correlation between *T. melanosporum* production and the size of 41 burns, with burn size explaining 26% of the variability in carpophore production. However, statistical analysis of the results indicates that average production of 21 burns with *Cistus laurifolius* is 73 % lower than the production of 20 burns associated with *Quercus/Corylus* without *Cistus laurifolius* in this zone, respectively. *C. laurifolius* develops small burns, which has an impact on their carpophore production, and their soils have 92 % less active carbonate than the burns associated with *Quercus/Corylus* in this zone, respectively. The low levels of active carbonate allow *Cistus laurifolius* to grow, but impair *Tuber melanosporum* production. Moreover, it has also been

statistically verified that many *Cistus laurifolius* die in *Tuber melanosporum* burns, whereas in old burns with minimum carpophore production, a new colonization of young *Cistus* has been observed. We thus provide a mechanism, based on the inability of *C. laurifolius* to grow in highly carbonated soils, which contradicts the well-known fact that *C. laurifolius* might act as a transmitter of *Tuber melanosporum* mycorrhizae. Consequently, the commercial cultivation of *Cistus laurifolius* mycorrhized with *Tuber melanosporum* for production is not recommended in truffle culture. Moreover, we reported negative observations of carpophore production in cultivations of *T. melanosporum* with other *Cistus* species.

CURRENT LINES AND IMPACT OF SPANISH RESEARCH IN INTERNATIONAL TRUFFLE KNOWLEDGE

It can be seen from the bibliography how the effects of the European Union's Common Agriculture Policy and reforestation policies in EU have led to a revival of interest in mycorrhizal plants and truffle cultivation since the 1990s. In Spain, this in turn has meant an increase in the number of nurseries which mycorrhize commercial plants. The Spanish Forestry Strategy [63], on which the Spanish Forestry Plan is based, underlines the importance of promoting research and development into the exploitation of *Tuber melanosporum*, *T. aestivum* Vittad. and other truffles in Spanish forests. However, these activities have given rise to new technical and economic problems in UE related to the mycorrhization of commercial plants, the appearance of contaminating fungi, the certification of commercial plants, the importation of Asian truffles, management of truffle-producing forests, and the need to optimise cultivation sites and repopulation efforts, among other aspects.

In addition, the yield of naturally-occurring truffles in Spain and other European forests appears to have decreased significantly in the last decade, making it necessary to study this trend in production more closely. Reyna [64] call attention to this problem and Garcia-Montero [65] describe how certain areas in central Spain shown a 35% decrease in their maximum annual production of *Tuber melanosporum* between 1960 and 2002. The decrease in production in this region appears to be associated with the depletion of various truffle-producing areas (due to over-harvesting), an increase in vegetation cover owing to changes in the use of the land (decrease in cattle-farming), and the fact that the average yearly rainfall fell by 35% between 1960 and 2000. The climatic effect appears to have been worsening in recent years.

Several lines of investigation have been opened in Spain in order to address these problems. The TESEO database [64], which contains a list of the doctoral theses written in Spain, describes six theses associated to the keyword "*Tuber melanosporum*", since 1994 to 2008. Moreover, a seventh doctoral thesis on truffle aroma is currently in the final stages; the first phase was completed by Diaz [66].

The doctoral thesis by Reyna [67] (directed by J. A. Rodriguez) proposes a set of models for use in forestry and the management of forest stands with *Tuber melanosporum* production. These models are developed taking into account social, economic and historical aspects, and are designed to

integrate truffle ecology, the state of tree stands and an improvement in forest yields by means of forestry techniques.

The doctoral thesis by Dominguez [68] (directed by J. A. Rodriguez) described in the TESEO data base [69] follows the same line of research, and is designed to increase the presence of *Tuber melanosporum* in forestry. This author studies the procedures for improving the mycorrhization of *T. melanosporum* and the use of this truffle in the recuperation of deteriorated ecosystems. To this end, studies have been done on how to improve the quality of the forest profile, the ecology of *T. melanosporum*, and the dynamic of its mycorrhizas. This author conducted tests with *Pinus halepensis* Mill., *P. nigra*, *Quercus ilex* L., *Q. faginea* Lam., and *Q. petraea* (Matt) Liebl. mycorrhized with *Tuber melanosporum*, and studied the effect of different substrates, inoculate and water stress on the efficiency of the mycorrhization and the quality of the plant.

He also analyses the influence of mycorrhizas on the biometry of the plant, nitrogen and phosphorous assimilation, gaseous exchange and water relations. He then locates potential areas for *Tuber melanosporum* in eastern Spain and uses cultivation plots to study the mycorrhizas and their contribution to the growth and physiology of *Quercus ilex* and *Q. faginea*. The author concludes that *Tuber melanosporum* improves biometry, survival, water availability in situations of stress, and nitrogen and phosphorous assimilation in plants; and he asserts that the host plant and the terrain are the two main factors determining the implantation of mycorrhizas, while the controlled presence of contaminating fungi from the nursery does not represent any significant competition for *T. melanosporum* in the short term. The first results of this thesis have been published in Dominguez [70].

Another line of research concerning the ecology of *Tuber melanosporum* can be found in the doctoral thesis by Garcia-Montero [41] (directed by J.L. Manjon) conducted in the Alto Tajo Nature Reserve (Guadalajara). This author propose new ecological data, such as the aforementioned importance of burn sizes, active carbonate in soils inhabited by *T. melanosporum*, the capacity of *Quercus faginea* and *Q. ilex* L. subsp. *ballota* (Desf) Samp. in truffle production and the difficulty some symbiont plants have in producing *Tuber melanosporum* sporocarps.

Another problem in Spanish truffle culture is the existence of mycorrhizas and fungi which are harmful to truffles in natural areas and in nurseries with mycorrhized plants. An example is the mycorrhiza known as "form 2" and the discomycete *Sphaerospora brunnea* (Alb. and Schwein. ex Fr.) Svrcek and Kubicka, which have been studied at the Universities of Alcala and Navarra and the Technical University of Madrid (UPM), together with other competing fungi. Regarding *S. brunnea*, Garcia-Montero [71] confirm that no appreciable damage has occurred in nurseries in central Spain which produce plants mycorrhized with *Tuber melanosporum*, in spite of the fact that it can clearly be seen to be fruiting in the containers of these plants.

A study of new *Tuber* mycorrhizas can be found in the doctoral thesis by Garcia-Montero [72] and Garcia-Montero et al. [70], that describe that the mycorrhizas of *T. pseudoex-cavatum* Wang, G. Moreno, L. G. Rioussset, J. L. Manjon and G. Rioussset, sp. nov., *T. indicum* Cooke and Masee and *T.*

malengonii Don., Riouss. and Chev. . These results agree with the observations of Amicucci , Zambonelli and Comandini and Pacioni . At the present time the authors of this article detect an increase in the presence (both legal and fraudulent) of Asian truffles in Spanish markets, and there is also a rumour (unconfirmed) circulating among European truffle farmers that plantations of plants mycorrhized with Asian *Tuber* spp. have already been introduced (either accidentally or deliberately), which could spread to the natural environment.

Also on the subject of mycorrhizas which are harmful to truffle culture is a doctoral thesis by Etayo (directed by A. M. De Miguel) described in TESEO . This author monitored a *Tuber melanosporum* plantation in northeast Spain in order to verify the permanence of its mycorrhizas and their interaction with other competing fungi. This author compares the behaviour of *Corylus avellana* L., *Quercus faginea* and *Q. ilex* subsp. *ballota*, and analyses the effect of mulching the ground with straw and plastic. This author identifies 14 morphotypes of mycorrhizas inside the cultivated area and 21 morphotypes in the area outside. Etayo demonstrates that the factor most influencing the mycorrhiza community is the tree species, while the type of mulch has less effect. This author also concludes that the symbiont plant which most favours the permanence of the mycorrhizas of *Tuber melanosporum* is *Quercus ilex* subsp. *ballota*.

A doctoral thesis by Roman (directed by A. M. De Miguel), described in TESEO , also deals with the subject of mycorrhizas and truffle culture. This author studied a mycorrhiza community associated with *Quercus ilex* subsp. *ballota* in a forest in the north-east of Spain for 3 years, in a potential area for *Tuber melanosporum* which had suffered a low-intensity forest fire four years earlier. This author describes and classifies 36 morphotypes of ectomycorrhizas and indicates that the mycorrhiza community is dominated by *Cenococcum geophilum* Fr. and by a specific group of morphotypes.

Roman also explains that the fire caused significant changes in the relative abundance of specific morphotypes and a decrease in the quantity of existing mycorrhized apexes, but it did not alter either the diversity or the specific composition of the mycorrhiza community. This author confirms that in the area affected by the fire, the mycorrhiza community is now in the process of recovery, although with a seasonal variation in mycorrhiza diversity which is highest in autumn and lowest in summer, while the quantity of mycorrhized apexes is highest in winter and lowest in autumn. The author asserts that 3 years after the introduction in the burn area of plants mycorrhized with *Tuber melanosporum*, it has been observed that the mycorrhizas of this truffle persist, although they have to compete with other mycorrhizal fungi. In conclusion, Roman proposes *T. melanosporum* as a suitable mycorrhiza for reforesting areas damaged by fire.

Finally, a doctoral thesis by Martinez de Aragon (directed by C. Colinas), described in TESEO , also deals with the subject of edible mushroom production, mycorrhizas ecology and truffle culture. This author studied the relationship among climate, forest management and productivity of edible mushrooms in 23 plots located in pine woods during 5 years. Moreover, this author analysed the fire effects in the mycorrhizal communities and he devoted particu-

lar attention to *Tuber melanosporum* cultivation in the burned areas.

Although there has been intense activity in Spanish truffle research over the last years, its influence on international truffle knowledge has just begun. Since 1994 to 2006, Spanish research has produced 200 references on truffles developed in 13 centres (6 doctoral theses, 67 papers in international scientific congresses, 49 papers in Spanish scientific congresses, 7 documents on websites, 63 published articles, and at least 8 papers submitted)

At 2007, as result of the research until 2006, the Spanish bibliography contains at least 15 papers published in international journals listed in the JCR: Moreno *et al.* [4] and Wang propose 2 new Asian species of *Tuber* (however, one of these be regarded as a synonym of *T. indicum*); Moreno-Arroyo provide new information on *T. oligospermum* (Tul. and C. Tul.) Trappe; Diaz have developed a rapid and objective methodology for differentiating truffles by means of the analysis of their aroma; Dominguez and Garcia-Montero

articles have been described above; and Suz propose a method using molecular biology to detect *Tuber* mycelium in the ground. A brief description is given below of the contributions and the international impact of these Spanish papers.

Moreno and Wang describe *Tuber pseudohimalayense* sp. nov. (recently be regarded as a synonym of *T. indicum*) and *T. pseudoexcavatum* sp. nov. These authors note the importation of Asian truffles which have been flooding the European markets. This is an opportunity to study species of *Tuber* which are practically unknown here, and although it has brought to light some taxonomic novelties, it has also caused serious damage to the quality of commercial truffles.

Tuber oligospermum is a poorly known species that has several synonyms. Moreno-Arroyo *et al.* [80] report on the taxonomy, habitat and biogeography of *T. oligospermum*, and they discuss its most important macroscopic and microscopic features. *T. oligospermum* is an edible truffle found only in the Mediterranean region; it is a circum-Mediterranean species, with an economic and ecological potential in developing regions (e.g. reforestation with mycorrhizal plants).

Several studies have been done in various countries on the subject of truffle aroma, and specifically on the aroma of *Tuber melanosporum*, *T. magnatum* Pico, *T. aestivum*, *T. borchii* Vittad. and other truffles of lower quality, when fresh, frozen and tinned . The techniques used for the extraction of the volatile compounds include both static and dynamic headspace analysis, headspace solid-phase microextraction (HS-SPME) , and extraction with organic solvents, among other techniques. The volatiles extracted are subsequently analysed by means of gas chromatography (GC) and/or gas chromatography coupled with mass spectrometry (GC-MS). These studies describe the key compounds in the aroma of *T. melanosporum* and *T. magnatum*. *T. magnatum* presents a characteristic compound, bis(methylthio) methane , while the aroma of *T. melanosporum* presents various compounds of interest which contribute to their total aroma . New developments in-

elude an "electronic nose" with various sensors which has been applied to *T. magnatum* and a patent portable truffle detector, however the "electronic nose" is still in the field of the research.

The bibliography and the various databases show that the studies on truffle aroma so far have used sporocarps from truffles of French and Italian origin. For this reason, Diaz

have applied experimental design and optimization techniques (response surface methodology) to develop an objective method for analysing Spanish truffle aroma, and they are studying the influence of several environmental and population factors of the truffles (intra and interspecific) on the aroma of *Tuber melanosporum* and other central European truffles. Finally, work has begun on the relationship between the volatile compounds of truffles and several flies of the genus *Suillia* and beetles of the genus *Leiodes* present in central Spain. Some publications have already analysed the relationship between dimethyl sulfide and *Suillia gigantea*, and between *S. univittata* (the garlic fly) and the aroma of *Tuber magnatum*, although nothing has yet been published on the compounds involved in these interactions.

Suz have developed a molecular method to detect mycelia of *Tuber melanosporum* in soil, and tested the mycelial distribution around 2 truffle-bearing *Quercus ilex* trees in a truffle orchard. Isolation of total DNA from soil was performed, followed by PCR amplification with *Tuber melanosporum* specific primers and restriction analysis. To address the detection sensitivity level, soil samples were inoculated with known amounts of gleba of *T. melanosporum*. Mycelium was detected primarily within the area defined by the truffle burn and within the top 35 cm of the soil in all directions from the trees.

Moreover, other important Spanish references are Bonet and Reyna [98]. Bonet have tested a method for quantifying irrigation needs for truffle plantations in the establishment phase based on water deficits. They proposed the use of calculating water needs as a function of water deficit in order to promote a rational method that can be adjusted to different sites and conditions. Reyna's [98] books are important on the popularization of the truffle culture in Spain, and are essential on the development of the truffle knowledge and research in Spain.

Therefore, Spain is one of the main truffle-producing countries in the world, and is of significant interest to international truffle culture due to its wide diversity of habitats for truffle study and cultivation. For these reasons, at least 13 research centers in Spain have been involved in investigation into truffle culture since 1994, and they have produced more than 217 references until 2007. This activity has shown how truffle research in Spain is now at the same level that other countries such as France. This documentation is intended to encourage scientific collaboration between Spain and other countries involved in truffle harvest and cultivation, such as Australia, New Zealand and USA.

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